Journal of Clinical Periodontology
Official Journal of The European Federation of Periodontology
Founded by the British, Dutch, French, German, Scandinavian, and Swiss Societies of Periodontology

Titanium implant functionalization with phosphate-containing polymers may favor in vivo osseointegration

Journal:	*Journal of Clinical Periodontology*
Manuscript ID	CPE-09-16-6565.R1
Manuscript Type:	Animal Experiment
Date Submitted by the Author:	n/a
Complete List of Authors:	Cardoso, Marcio; KU Leuven, Department of Oral Health Sciences & Dental Clinic; Biomaterials – BIOMAT & Restorative Dentistry de Rycker, Judith; KU Leuven, Department of Oral Health Sciences & Dental Clinic; Biomaterials – BIOMAT & Restorative Dentistry Chaudhari, Amol; KU Leuven, Department of Oral Health Sciences & Dental Clinic; Biomaterials – BIOMAT & Restorative Dentistry Coutinho, Eduardo; Universidade Federal Fluminense Yoshida, Yashuhiro; Hokkaido Daigaku Shigakubu Daigakuin Shigaku Kenkyuka, Department of Biomaterials and Bioengineering Meerbeek, Bart; KU Leuven, Department of Oral Health Sciences & Dental Clinic; Biomaterials – BIOMAT & Restorative Dentistry Mesquita, Marcelo; KU Leuven, Department of Oral Health Sciences & Dental Clinic; Biomaterials – BIOMAT & Restorative Dentistry; Piracicaba Dental School, Prosthodontics and Periodontology da Silva, Wander; KU Leuven, Department of Oral Health Sciences & Dental Clinic; Biomaterials – BIOMAT & Restorative Dentistry; Piracicaba Dental School, Prosthodontics and Periodontology Yoshihara, Kumiko; Okayama Daigaku Byoin, Center for Innovative Clinical Medicine Vandamme, Katleen; KU Leuven, Department of Oral Health Sciences & Dental Clinic; Biomaterials – BIOMAT & Restorative Dentistry Duyck, Joke; KU Leuven, Department of Oral Health Sciences & Dental Clinic; Biomaterials – BIOMAT & Restorative Dentistry
Topic:	Implantology
Keywords:	functionalization;, osseointegration, pullulan, surface, implant
Main Methodology:	Animal Model

Titanium implant functionalization with phosphate-containing polymers may favor *in vivo* osseointegration

Marcio V Cardoso[1], *Judith de Rycker*[1], *Amol Chaudhari*[1], *Eduardo Coutinho* [1,2], *Yasuhiro Yoshida*[3], *Bart Van Meerbeek*[1], *Marcelo F Mesquita*[1,4], *Wander J da Silva*[1,4], *Kumiko Yoshihara*[5], *Katleen Vandamme*[1], *Joke Duyck*[1]

[1] Department of Oral Health Sciences & Dental Clinic; Biomaterials – BIOMAT & Restorative Dentistry; KU Leuven & University Hospitals Leuven, Leuven, Belgium

[2] School of Dentistry, Fluminense Federal University, Brazil

[3] Department of Biomaterials, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

[4] Department of Prosthodontics and Periodontology, Piracicaba Dental School, State University of Campinas, Piracicaba, Brazil

[5] Center for Innovative Clinical Medicine, Okayama University Hospital, Okayama, Japan

Running Title: Functionalizing Ti for osseointegration

Keywords: functionalization; implant; osseointegration; pullulan; surface

Conflict of Interest and Sources of Funding Statement:

- The authors declare that there are no conflicts of interest in this study.
- This study was funded by the KU Leuven Research Fund, grant number OT06/55

Correspondence to:
Prof. Dr. Joke Duyck
KU Leuven & University Hospitals Leuven;
Department of Oral Health Sciences & Dental Clinic;
Biomaterials – BIOMAT & Restorative Dentistry
Kapucijnenvoer 7 - Blok a - Bus 7001; 3000 Leuven, BELGIUM
Tel.: +32 16 33 24 68; Fax: +32 16 33 23 09
Joke.Duyck@uzleuven.be

ABSTRACT

Aim: Osseointegration of titanium implants is predictable, but can be improved via surface functionalization.

Materials and Methods: 120 implants were installed in parietal bone of 12 domestic pigs and left to heal for 1 or 3 months. Five groups were defined according surface treatments: immersion in water (H2O), 10% polyphosphoric acid (PPA10), 1% phosphorylated pullulan (PPL1), 10% phosphorylated pullulan (PPL10), or 10% phosphorylated pullulan + 1 µg BMP-2 (PPL10 BMP). As primary outcome, implant osseointegration was evaluated by quantitative histology, namely peri-implant bone formation (B/T in %) and bone-to-implant contact (BIC in %) for each healing period. The Wilcoxon signed-rank test and Mann Whitney U-test with α=0.05 were performed.

Results: PPL10 and PPA10 groups showed significantly higher B/T and BIC results than the control (H2O) group at 1-month ($p<0.05$). No significant difference was found between PPL1 and H2O or between PPL10 BMP and H2O, irrespective of healing time (1 or 3 months) or investigated parameter (B/T and BIC; $p>0.05$). After 3 months, no experimental group showed a significant difference compared to the control group (H2O) for both investigated parameters (B/T and BIC; $p>0.05$).

Conclusion: Functionalizing titanium implants with inorganic or organic phosphate-containing polymers at 10 wt% concentration may stimulate peri-implant bone formation and implant osseointegration at early healing times.

CLINICAL RELEVANCE

Scientific rationale for the study: To investigate the influence of different implant surface treatments on the efficiency of bone regeneration in a simulated clinical situation.

Principal findings: Both PPA10 and PPL10 seem to induce faster peri-implant osseointegration and bone regeneration. Use of PPL as a carrier for BMP-2 was not efficient on stimulating peri implant bone formation.

Clinical implications: PPA10 and PPL10 may promote more favorable conditions for early implant loading, particularly in unfavorable clinical situations.

ACKNOWLEDGEMENTS

This study was funded by the KU Leuven Research Fund, grant number OT06/55.

INTRODUCTION

Aiming the enhancing of the osseointegration, implant surface modifications were developed over the years (Steigenga et al. 2003, Wennerberg & Albrektsson 2010). Different implant surface treatments have been investigated (Taxt-Lamolle et al. 2010, Wennerberg & Albrektsson 2010). Bioactive functionalization of the titanium surface by chemical modifications is some of these (Liu et al. 2007, Choi et al. 2012, Jimbo et al. 2012). Especially when the protocol of rehabilitation is anticipated, a fast and effective osseointegration is necessary. Whenever the loading of the implant is required at early stages of peri-implant bone healing, a faster tissue regeneration is desirable to assure a more predictable clinical outcome. This becomes particularly important in less favorable bone conditions which are usually related to systemic disorders or implantation-site defects. By improving the peri-implant tissue regeneration, some common clinical problems could be

avoided. Unfortunately, this remains a challenge for clinicians and researchers (Esposito et al. 2013).

The functionalization of implants with fluoride has been studied and is commonly used in clinical practices (Steigenga et al. 2003, Mertens & Steveling 2011, Choi et al. 2012). Despite showing a positive effect on the osteoconductivity of titanium, functionalization with calcium phosphate has been only experimentally investigated (Paital & Dahotre 2009), due the presence of some inconveniences, as non-uniformity in crystallinity and morphology, low mechanical properties and poor adherence to the substrate (Paital & Dahotre 2009), limiting its clinical application. Recently, a simple and cost-effective treatment has been suggested for the surface functionalization of titanium implants by coating them with an inorganic phosphate polymer, namely polyphosphoric acid (PPA). This treatment seems to provide promising results on bone-like cell attachment, proliferation and differentiation (Maekawa et al. 2007) and on the promotion of direct bone bonding to titanium (Maekawa et al. 2009).

It is well-known that phosphates are present in human osteoblasts, indicating a possible correlation between bone cell differentiation and phosphate groups (Leyhausen et al. 1998). It has been demonstrated that polyphosphates induce maturation and calcification of bone-related cells (Kawazoe et al. 2004, Gopalakrishnanchettiyar et al. 2009). In *in vitro* conditions, the surface phosphorylation of biomaterials has been considered as a potential technique to impart biomimetic nucleation and mineralization of calcium phosphate on biocompatible surfaces (Anselme et al. 2000, Sailaja et al. 2009) thus providing preferential binding sites for bone cells.

Besides inorganic phosphate polymers, the use of an organic biopolymer, pullulan, has been successfully applied in bone tissue engineering (Cheng et al. 2007, Kato et al. 2007, Fujioka-Kobayashi et al. 2012). Pullulan is an exocellular homopolysaccharide produced by the fungus *Aureobasidium pullulans* (Prajapati et al. 2013) and it has been studied as food

additives to environmental remediation agents (Cheng et al. 2011). The use of pullulan in biomaterial research is justified by its proven biocompatibility, drug delivery properties (Morimoto et al. 2005, Kato et al. 2007, Prajapati et al. 2013), cell stimulation (Thébaud et al. 2007) and unique linkage pattern, including adhesive ability and the capacity to form thin and transparent biodegradable films (Prajapati et al. 2013). Moreover, another important advantage of pullulan over polyphosphoric acid is its ability to effectively act as a carrier to growth factors for bone tissue engeneering (Fujioka-Kobayashi et al. 2012).

Pullulan presents hydroxyl groups which can easily be substituted by grafting different chemical groups (Prajapati et al. 2013). In that respect, Cardoso et al. (2014) have described a method for phosphorylation of pullulan (PPL) and showed osteogenic-promoting results by treating titanium surfaces with a solution of 10 wt% phosphorylated pullulan. However, macro-design of dental implants, insertion torque, achievement of implant stability and the presence of trabecular bone structure - important clinical aspects - were not taken into account, thereby urging the need for a follow-up study in a larger animal model in which most clinically relevant aspects could be taken into account. For this purpose, implantation of functionalized threaded implants in the bone of swine has been employed taken into account the similarities with human beings in terms of bone anatomy and metabolism (Pearce et al. 2007). Subsequently, well-stablished histomorphometrical analyses can be carried out based on quantitative observations, namely bone density and bone-to-implant contact, both equally important aspects for the stabilization of implants under functional loading (Lutz et al. 2008, Cardoso et al. 2014).

Considering the functionalization of implants with phosphate-based polymers as an alternative approach to foster bone healing processes (Cardoso et al. 2014) and the potential of pullulan to serve as a carrier to growth factors for bone engineering (Fujioka-Kobayashi et al. 2012), this study aimed to evaluate the influence of PPA and PPL on peri-implant bone

regeneration, the latest being produced at different concentrations and once adsorbed with a specific osteoinductive protein, namely bone morphogenetic protein 2 (BMP2). It has been hypothesized that the functionalization of the implant surface with phosphate-based polymers could accelerate and improve bone regeneration and that this improvement could be maintained over time.

MATERIALS AND METHODS

Animal model

This study was approved by the Ethical Committee for Laboratory Animal Research of the Catholic University Leuven (P141-2009). Animals were handled according to the Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (86/609/EEC) and the Royal Belgian animal welfare regulations and guidelines. 12 six-month old female domestic pigs (*Sus scrofa domestica*, Collaert, Hoegaarden, Belgium) were employed. Under circadian day and night rhythm, they were housed on straw at favorable conditions of temperature and humidity. Food (standardized pig mast fodder, Garant Tiernahrung GmbH, Pöchlarn, Austria) and water were supplied ad libitum. Their weigh ranged from 103 to 124 kg at the moment of the surgical procedure. All animals were observed throughout the study by a veterinarian and a veterinary technician to assure appropriate health conditions. The study has been carried out from February/2010 to February/2011. The animal experiment (period between the surgical intervention of the first animal and sacrifice of the last animal) occurred between early June and early August/2010. During this period, all efforts were considered to minimize suffering and no pathologic signs or symptoms were observed. This manuscript followed the ARRIVE guidelines for reporting animal research (Kilkenny et al. 2010).

Sample size was estimated according to previous literature and experience. International standards stablish that at least 2 animals should be used for each treatment at each healing time when implantation of biomaterials in bone of pigs is considered (Pearce et al. 2007). Previous research using the same methodology and implantation site (Lutz et al. 2008) showed that the use of 4 animals were sufficient to assure statistical significance. To compensate possible drop-outs, while minimizing the number of animals, the sample size in the present study was estimated to be n = 6, i.e. 12 animals in total. This number has been confirmed to be sufficient to assure statistical significance in our previous research in which the same methodology was employed (Cardoso et al. 2017).

Implants

Sixty custom-made titanium implants were machined to present 2 threaded parts, each 3 mm in length and with an outer diameter of 1.8 mm, separated by a non-threaded groove of 1 mm in length and a diameter of 1.1 mm (Fig. 1A and 1B). The threaded part was designed to be in contact with the host bone (immediate bone-to-implant contact) while the grooved part was designed to be kept away from the walls of the surgical site (no immediate bone-to-implant contact), thus defining two regions of interest to be separately evaluated: groove (G) and thread (T). After machining, all implants were cleaned with acetone in an ultrasonic bath, washed with distilled water for three times and acid-etched in a solution containing 4 vol% hydrofluoric acid (HF 40%; Riedelde Haen, Heverlee, Belgium) and 20 vol% nitric acid (HNO_3 65%; ChemLab, Zedelgem, Belgium) at room temperature for 60 s. Implants were then washed three times in distilled water and taken for standard autoclave sterilization in individual titanium containers.

Implants were then divided into 5 groups defined by 5 different surface treatments, namely immersion in distilled water (H2O, control group), 10% polyphosphoric acid

(PPA10), 1% phosphorylated pullulan (PPL1), 10% phosphorylated pullulan (PPL10), or 10% phosphorylated pullulan + 1 µg BMP-2 (PPL10 BMP). In the control group, implants were simply stored in distilled water for 24 h at 37°C. Treatment of the implants with PPA10 was done by immersion in 10 wt% PPA solution for 24 h at 37°C. This solution was obtained by dissolving 10 g of 100% PPA (Merck Schuchardt, Hohenbrunn, Germany) in 100 ml of distilled water. For all groups treated with PPL (PPL1, PPL10 and PPL10 BMP), phosphorylated pullulan solutions were obtained by dissolving PPL powder in sterilized distilled water in concentrations of 1 wt% or 10 wt%. In order to increase the reactivity of the polymer, PPL solutions had their pH assessed and lowered to pH 5 by gradual addition of hydrochloric acid 0.1M until the specified pH was reached. They were then filtered using a GD/X polyethersulfone 0.2 µm filter (Whatman, Maidstone, UK). After treatment with PPA10, PPL1 or PPL10, samples were washed in demineralized water and air dried for 20 min at room temperature. In the group PPL10 BMP, samples were initially treated with 10 wt% PPL solution as described above. Subsequently, 10 µL of a 10% BMP2 solution was applied on each implant surface for the adsorption of 1 µg of the mentioned protein. This solution was obtained by re-constituting 10 µg of BMP-2 (RD Systems, Minneapolis, MN, USA) in 100 µL of phosphate-buffered saline (PBS) according to manufacturer's instructions. To maintain the bioactivity of the protein, the adsorption of 10% BMP2-PBS solution onto the PPL-treated surfaces was carried out shortly before the surgery. Care was taken to avoid contamination of the protein and to keep the environmental temperature constant to avoid protein denaturation. The synthesis of PPL was processed as previously described in detail by Cardoso et al. (2014). All treatment steps were performed under sterile conditions.

Four additional titanium plates were prepared and treated with H2O, PPA10, PPL1 or PPL10 as described above. These samples were used for spectrophotometric analysis of the colorimetric reaction of the phosphate-molybdenum compound (Han et al. 2016) in order to

evaluate the amount of phosphate-containing polymer on the treated titanium surfaces. For molybdenum blue measurement, the specimen were immersed in 20 ml of 1 wt% sulfuric acid solution for 30 minutes at 70°C. The solution in which each specimen was immersed was corrected for molybdenum blue measurement. The phosphoester compound in the sample solution was decomposed with potassium persulfate in autoclave. Concentration of resulted phosphate ions in the supernatant liquids were then measured via molybdenum blue reaction. For the molybdenum blue reaction, 0.6 g of ammonium molybdate tetrahydrate and 0.024 g of potassium antimony tartrate hydrate were dissolved in 30 mL deionized water. To produce the color reagent solution, 10 mL of 7.4 M concentrated sulfuric acid, 10 mL of 0.44 M ammonium sulfamate, and 10 mL of 0.4 M L-ascorbic acid were added. Subsequently, 1 mL of the color reagent solution was added to 6 mL of the sample solution. The absorbance of the molybdenum blue was recorded using a spectrophotometer (U-1900, Hitachi High-Technologies Corporation, Tokyo, Japan) over time or read at 883 nm after 30min. For each sample, duplicate measurements were performed.

Surgical protocol

The animals were pre-anesthetized with 1 mL of butyrophenone i.m. (Stresnils®, Ausrichter, Annandale, Australia) and anesthetized by intravenous injection of a premixed combination of tiletamine and zolazepam (Zoletil 100®, Virbac, Barneveld, the Netherlands), added at 1/6 dilution to Xylazine (Vexylans, CEVA, Brussels, Belgium), and injected at 1 mL/10 kg. The anesthesia was maintained by isoflurane at 1.5% concentration (IsoFlos, Abott, Quebec, Canada). After applying local anesthetic subcutaneously at the surgical site (Lignospan, Septodont, Cedex, France), a sagittal incision was made and the soft tissues and periosteum were mobilized. Five implantation sites were drilled in a transversal sequence in the parietal bones 0.6 mm far from the coronal suture (Fig. 1C and 1D). Two of these sites

were drilled on the right parietal bone while the other three on the left parietal bone, always keeping a distance of 12 mm between adjacent drilling sites. The drilling sequence (speed of 1500 rpm with water cooling) was: (1) guide drill (round bur); (2) twist drill (diameter of 1.2 mm); (3) twist drill (diameter of 1.5 mm) and (4) twist drill (diameter of 1.7 mm). This resulted in a difference of 0.1 mm between the diameter of the final drill and the implant. Five implants, one of each of the five groups described above, were installed. Sites of implantation were randomly allocated to experimental groups using a computer-generated list (www.randomization.com) with a 1:1 allocation and random block size of 5. At the end, soft tissue flaps were repositioned and sutured. All surgical procedures were performed by the same calibrated operator. Postoperatively, ibuprenorfine i.m. (0.005mg/kg; Temgesic, Schering-Plough, Brussels, Belgium) was administered as analgesic and enrofloxacin i.m. (0.5ml/10kg; Baytril 5%, Bayer, Puteaux, France) as antibiotics for 3 days.

Half of the animals (six) were randomly selected and euthanized after 1 month (1-month group) while the remaining ones were euthanized 3 months post-implantation (3-months group), through sedation induction by i.m. injection of 1 mL of butyrophenone (Stresnils®, Ausrichter, Annandale, Australia) followed by an intravascular injection of an embutramide–mebenzoniumjodide–tetracaıne-HCl solution (1mL/5kg; T61, Intervet, Mechelen, Belgium) into the ear vein until cardiac arrest occurred. It is noteworthy that a second implant per group was also inserted in the frontal bone during the surgical procedure but these were not used due to the extensive pneumatization of the frontal bone at the time of sacrifice.

Specimen preparation and analysis

The implant with the surrounding skull bone tissue were harvested, fixed for 4 days in a $CaCO_3$-buffered formalin solution and dehydrated in an ascending series of ethanol concentrations over a period of 15 days. Embedding was performed by infiltration of a

benzoylperoxide (0.018%)-methylmetacrylate solution. Samples were sectioned (long axes) using a precision diamond saw (Leica SP 1600, Leica Microsystems, Nussloch, Germany). The most central section was micro-grinded, polished to a final thickness of 20 to 30 µm (Exakt 400 CS, Exakt Technologies Inc., Norderstedt, Germany) and stained with a combination of Stevenel's blue and Von Gieson's picrofuchsin red. Therefore, one single sample was evaluated per experimental group. All histological and histomorphometrical analyses were performed by the same blinded and calibrated examiner. Histological examination was performed using a light microscope (×40, ×100 and ×400 magnification; Leica Laborlux, Wetzlar, Germany). The images were captured using a high-sensitivity colour video camera (JVC TK-1280E, Ibaraki-ken, Japan). The assessment of the histomorphometrical data was performed using a commercially available semi-automatic image analysis software program (Axiovision 4.0, Zeiss, Gottingen, Germany), with an additional customized script (Ogawa et al., 2011).

Histomorphometrical analyses were performed for 2 implant regions, *i.e.* the grooved (unthreaded) [G] and the threaded lower [T] parts. Measurements were performed at both implant sides on each histological section (Fig. 2):

- Peri-implant bone tissue (B) relative to all tissues (T) (B/T, %) or bone density: the amount of bone in the peri-implant region up to 100 µm distance from the implant surface (Fig. 2A+B);

- Bone-to-implant-contact (BIC, %): summation of the lengths of contact between bone and implant/implant length under consideration (Fig. 2C).

The primary outcome was defined as the efficiency of different titanium surface treatments (H2O, PPA10, PPL1, PPL10, PPL10 BMP) on bone regeneration at 2 specific healing times, namely 1 and 4 months. The primary outcome variable was bone density (B/T in %) and bone-to-implant contact (BIC in %) within the two regions of interest, i.e groove

(G) and thread (T). The secondary outcome was to define the efficiency of these surface treatments on bone regeneration over time (1-month vs 3-month healing times).

Statistical analyses

The animal was defined as the statistical unit (n=6). Diagnostic tests comprising normal probability plots and Shapiro-Wilk tests (software package Statistica; Stat Soft 7.1, Tulsa, Oklahoma, USA) were employed to evaluate the homogeneity of the variance and to determine whether the residual errors were distributed according to the Gaussian curve. As the data did not meet the rigor required by a parametric evaluation, non-parametric tests were employed. The Wilcoxon signed-rank test was performed at a significance level of 5% to compare pairs of data sets originated from different surface treatments (H20, PPA, PPL1, PPL10 and PPL10 BMP) in a particular healing time (1 or 3 months) and a particular area of interest (G or T) for each of the selected parameters (B/T and BIC; primary outcome). It considers the dependency between pairs of data sets as they were always collected from the same animals. The results obtained for the 1 month and the 3 months groups were also compared (secondary outcome) using a non-parametric test for independent samples as pairs of data sets under comparison were collected from different animals. In this case, the Mann Whitney U-test was used with a significance level of 5%. Multiple testing was used to increase the sensitivity of the statistical analysis as the global number of comparisons was too large.

RESULTS

Spectrophotometric Measurement

The concentration of phosphate was determined by the absorption spectra in molybdenum blue solution. No absorption peak was noticed for samples treated with H2O (control) while

clear absorption peaks were observed when samples treated with PPA10, PPL1 and PPL10 were evaluated. The absorbance values for PPA10, PPL1 and PPL10 were 42 x 10^{-3}, 6 x 10^{-3} and 222 x 10^{-3} respectively.

Histological findings

In one animal (1-month group), signs of inflammation and fibrous tissue invasion at the healing sites were observed. These implants were excluded from analysis.

Bone healing at the implant site occurred intramembraneously and no cartilage tissue was observed. After 1 month, ongoing bone formation could be observed with cubical-shaped osteoblasts close to the implant surface and areas of new, non-mineralized bone tissue. In the group PPL10 BMP, a considerably higher presence of osteoclasts on the implant surfaces could be observed (Figure 3). After 3 months, the peri-implant tissue was more organized and the prevalence of mineralized bone tissue was observed. Also signs of remodeling, with osteoclast activity and the occurrence of basic multicellular units were seen. Figure 4 shows ten representative images of the areas of interest (G and T), being one per experimental group (H2O, PPA10, PPL1, PPL10 and PPL10 BMP) at each healing time (1 and 3 months).

Histomorphometrical findings

Results of peri-implant bone formation (B/T) and results of bone-to-implant contact (BIC) are and graphically presented in Figures 5 and 6 respectively. These results are also descriptively presented in Table 1 including means, medians and standard deviations. For each parameter, data are presented per area of interest, namely for the groove (G; Figures 5A and 6A respectively) and for the lower screw thread (T; Figures 5B and 6B respectively), and this for both 1-month and 3-month healing periods.

Significant differences were present for the peri-implant bone formation (B/T) among the different surface treatments and the control group ($p<0.05$). After 1 month, the implants treated with PPA10 and with PPL10 showed a significant larger amount of encasing bone at the implant groove level compared to the implants in the control H2O group (Fig. 5A; $p<0.05$). The same results were obtained for the implant threaded part ($p<0.05$), but a difference was also found between the implants functionalized with PPA10 and PPL10 when compared to the ones incorporated with BMP-2 (PPL10 BMP; $p<0.05$). The latter showed a significant lower amount of bone tissue (Fig. 5B). After 3 months of healing, no statistically significant difference was found between control (H2O) and experimental groups (PPA 10, PPL1, PPL10 and PPL10 BPM), irrespective of the area of interest (groove or thread; $p>0.05$). At the level of the screw threads (T), on the other hand, a significant difference was observed between the PPA10 group and the PPL10 BMP group ($p<0.05$).

Significant differences for BIC were found at the implant groove among different surface treatments after 1 month (Fig. 6A), with higher BIC values for PPA10 and PPL10 compared to the control H2O group ($p<0.05$). This difference was, however, not observed after 3 months ($p>0.05$). Furthermore, more BIC was observed in the groove region of the PPA10 implants compared to the PPL10 BMP group after 1 month ($p<0.05$). Contrary to the peri-implant bone formation, some significant differences in BIC between the groups persisted over time (3-month groups), in particular between the PPA10 and PPL10 BMP groups in the groove area ($p<0.05$). Additionally, significantly higher BIC values for PPL10 implants was noted as compared with the BMP-functionalized PPL10 implants (PPL10 BMP group) in the groove area at 3-month ($p<0.05$). With regard to the threaded implant part (Fig. 6B), significant more BIC was found for the PPA10 and PPL10 groups, compared to H2O group ($p<0.05$), but this was not sustained over time ($p>0.05$).

When results of B/T and BIC were compared over time (1 vs 3 months of healing), no statistically significant difference was found ($p>0.05$) irrespective of the surface treatment employed (H2O, PPA 10, PPL1, PPL10 and PPL10 BPM) or the region of interest evaluated (groove or thread).

DISCUSSION

Functionalization of the surface of implants is an actual trend in order to improve the integration with biological tissues (Taxt-Lamolle et al. 2010, Wennerberg & Albrektsson 2010, Cardoso et al. 2014). This study showed that both inorganic and organic phosphate-containing polymers favor early bone healing surrounding titanium implants, the latter in a dose-dependent manner. PPL is supposed to produce a thin coating onto the titanium surface, representing a possible alternative approach to foster bone tissue repair and potentially engineering.

A pig animal model was used to simulate the osseointegration in its full complexity (Chaudhari et al. 2012, Cardoso et al. 2014). Domestic pigs show considerable bone similarities to human beings, including important aspects such as osseous macrostructure, microstructure, composition and bone remodeling process and rate, therefore representing a suitable option for studies involving implants (Pearce et al. 2007). The calvaria was chosen due to its structural similarity to the maxillary region while allowing for a more controllable and reproducible surgical procedure when compared to protocols involving intraoral surgery (Lutz et al. 2008). Although *in vitro* testing is an alternative option, the chosen model allowed us to demonstrate the behavior of phosphate-containing polymers in a real bone repair situation in which not only biological aspects are taken into account but also mechanical issues related to the resistance of the coating against the mechanical challenges imposed by bone hard tissues while the implant is screwed into the surgical site. In this sense, implants

used in this study were customized to provide responses in two different sites. The grooved site represented an area of bone regeneration, completely devoid of contact with the walls of the bone surgical site (bone defect) at the moment of implantation. On the other hand, the threaded site represented an area of initial bone-to-implant juxtaposition (favorable clinical situation).

Implants treated with PPA10 and PPL10 showed higher values of bone formation (B/T) and implant osseointegration (BIC) when compared to the non-treated implants (H2O) after 1 month irrespective of the site to be considered (T or G). These results confirm the osteoconductive potential of both inorganic and organic phosphate-containing polymers as previously suggested by Cardoso et al. (2014), and this irrespective of the mechanical challenge during the insertion of the implant in the surgical site. From a histological and biochemical point of view, polyphosphate polymers seem to enhance the attachment and proliferation of osteogenic cells (Maekawa et al. 2009) besides inducing their maturation and calcification by accelerating alkaline phosphatase and osteocalcin gene expressions (Gopalakrishnanchettiyar et al. 2009, Kawazoe et al. 2004). An increase in alkaline phosphatase expression shows that the osteoblast activity has progressed to a more differentiated state (Anselme et al. 2000). Osteocalcin, in the same context, is a late marker associated with extracellular matrix mineralization and is produced by mature osteoblasts during bone tissue mineralization. Moreover, superficially adsorbed phosphate groups act as specific sites for nucleation of calcium phosphate, which offers preferential binding sites for bone cells (Anselme et al. 2000, Sailaja et al. 2009). Finally, Muller et al. (2015) has recently shown that polyphosphate stimulates osteoclast-like cells by promoting a significant increase in the levels of intracellular and extracellular ATP, thus functioning as a “metabolic fuel” for hydroxyapatite formation on the plasma membranes of osteoblasts.

Despite all the positive effects of phosphate groups on cell attachment, proliferation and differentiation, it should be considered that cell behavior and consequently osseointegration can also be greatly influenced by the morphology, roughness and hydrophilicity of the implant surface. A hierarchical surface topography including both micron-scale and submicron-scale structures seems to be able to promote an enhanced osteogenic effect (Zinger et al. 2005). In our study, a machined implant surface devoid of complex topographic structures has been employed to avoid that such physical aspects would overshadow the effect of the chemical surface modifications, which represent the main object of this study. As previously presented, no topographical modification can be expected after treatment of titanium surfaces with PPA and PPL in either micro or nanometrical level (Cardoso et al. 2014), suggesting that all samples employed displayed the same morphology and roughness features irrespective of the treatment to which they were submitted. On the other hand, wettability has also been reported to influence the interaction between the implant surface and the biological environment (Buser et al. 2004). In this sense, a possible increase in hydrophilicity promoted by the treatment of the implants with PPA and PPL (Cardoso et al. 2014) may have played a role on the positive effect of these treatments on bone formation and osseointegration.

The data also showed that the positive results induced by PPA10 and PPL10 after 1 month of healing did not persist over time. After 3 months, no statistically significant difference was found between H2O and PPA10 or PPL10. This suggests that such treatments will not necessarily improve osseointegration in a long term, but they are able to accelerate bone formation in earlier healing stages. Such faster regeneration is highly desirable as it may shorten unloaded healing periods, besides rendering immediate loading protocols more successful and predictable. It is indeed in the early stages of bone regeneration that bone-implant integration is more susceptible to loading challenges (Gapski et al. 2003). A faster

regeneration may also be useful in cases of low bone quality due to compromised systemic conditions or site-related bone defects.

It is noteworthy that no difference was found between PPL1 and the control group H2O in terms of bone density and BIC irrespective of the area of interest (G or T), which suggests that the positive effect of phosphorylated pullulan may increase in a concentration dependent manner. Indeed, our Spectrophotometric measurements showed that treatment with PPA10 leads to a noticeably higher adsorption of phosphate groups on titanium than its lower concentration version (PPA1). This is in line with a previous study in which a higher phosphate-titanium ratio tended to accelerate the proliferation of human bone marrow derived mesenchymal stem cells (Maekawa et al. 2009). In general, the threaded surface attained higher B/T and BIC values compared to the unthreaded implant part, in particular for BIC. An increased surface area for the cells to adhere to when initiating bone healing and a tissue interlocking more resistant to micro-movements may explain the positive effects of the screw-shaped implant design.

Thanks to its unique linkage pattern, pullulan offers interesting physical properties, including adhesive ability and the capacity to form thin and transparent biodegradable films (Cheng, Demirci & Catchmark, 2011). On the other hand, pullulan films are highly soluble (Tong, Xiao & Lim, 2008), leading to a fast release of phosphorylated polymers into the regeneration area. Despite its high solubility and consequent fast release, it has been shown that phosphorylate polysaccharides remain present on the surface of titanium treated with PPL even after active washing and storage in water (Cardoso et al. 2017), which gives us some insight on the stability of the coating. The Spectrophotometric measurements in this study also confirm the stable adsorption of phosphate-containing polymers on titanium in a concentration dependent manner, i.e. higher concentrations of PPL did lead to a higher adsorption on the treated surface. However, it remains unknown if the positive effect of PPL

is due to the phosphorylated polysaccharides which are released in the regeneration area, or due to the PPL that remains on the titanium surface as both aspects may be playing a simultaneous role. Moreover, it is still questionable if the film-building characteristic of PPL had any specific positive effect on bone regeneration and osseointegration. As no statistically significant difference was found between PPA10 and PPL10, their positive effect on bone tissue activity can only be ascribed to the stimulating effect of phosphate groups.

The potential use of PPL as carrier for bioactive agents was evaluated via the adsorption of 1 µg of BMP2 onto the implant surface functionalized with 10 wt% phosphorylated pullulan (PPL10 BMP). The results revealed that BMP2 was not able to improve the healing process and even negatively affected the late implant osseointegration (i.e. 3 months healing period) at the unthreaded implant part in terms of bone-to-implant contact when compared to PPL10. This may at first seem surprising as BMPs are reported to be potent osteoinductive morphogens capable of stimulating bone healing (Cheng et al. 2003). However, it has also been reported that BMP can exert a negative effect on bone formation (Wikesjö et al. 2008) as it may play a role in osteoclastogenesis as receptors for BMPs are also expressed in osteoclasts (Kaneko et al. 2000). Although osteoclast activity is inherent in the process of bone healing around implants, large doses of BMP-2 may lead to extensive bone re-modelling with consequent and undesirable bone loss (Wikesjö et al. 2008, Chaudhari et al. 2012). Unfortunately, only limited information is available regarding bone resorption due to a particular BMP-2 release via protein delivery carriers (Chaudhari et al. 2012). Additional studies are necessary to investigate the most adequate doses of BPM-2 to be adsorbed onto PPL-functionalized implants. Alternatively, further modifications in the properties of pullulan may be necessary for its use as a BMP-2-release carrier aimed at bone regeneration applications. The modifications should aim to a release profile which would induce a bone anabolic effect in a consistent, controlled and reproducible manner.

CONCLUSION

Phosphate-containing polymers, whether in their inorganic or organic form, may induce extended peri-implant bone formation and osseointegration in early phases of the healing process in a dose-dependent manner. Both PPA 10 wt% and PPL 10 wt% promoted higher values of B/T and BIC at 1-month healing time. After 3 months of healing, however, PPA and PPL seem not to play a role on histomorphometrical outcomes. The association of PPL10 and BMP-2 was not successful on promoting improved bone formation and implant osseointegration as far as the concentrations and conditions defined in this study are taken into account.

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TABLE

Table 1. Histomorphometrical results for Bone Fraction (B/T, in %) and Bone-to-implant Contact (BIC, in %)

Parameter	Area of Interest	Healing Time	*Mean ± Standard deviation* *Median (Minimum value - Maximum value)*				
			H2O	***PPA10***	***PPL1***	***PPL10***	***PPL10 BMP***
B/T (%)	**Groove**	**1 month**	29.2 ± 13.7 34.0 (5.5 - 40.6)	41.5 ± 9.2 41.0 (31.3 - 53.6)	36.3 ± 16.4 35.3 (19.0 - 54.9)	48.5 ± 7.7 47.4 (39.7 - 58.6)	37.7 ± 13.7 35.3 (23.1 - 59.0)
		3 months	45.5 ± 16.5 48.6 (21.3 - 66.6)	54.6 ± 15.4 54.5 (31.8 - 79.3)	43.6 ± 10.3 39.8 (35.3 - 62.7)	51.4 ± 12.7 57.7 (32.8 - 63.3)	38.8 ± 7.6 40.3 (25.5 - 45.7)
	Thread	**1 month**	48.3 ± 7.5 48.0 (38.7 - 58.8)	61.6 ± 10.1 58.8 (48.1 - 72.5)	59.0 ± 9.4 62.4 (43.2 - 67.3)	60.5 ± 3.1 59.5 (57.3 - 64.7)	52.3 ± 9.2 54.4 (40.6 - 64.5)
		3 months	61.5 ± 12.9 56.5 (51.6 - 85.8)	71.8 ± 8.0 71.3 (63.6 - 84.4)	63.2 ± 13.2 59.7 (46.2 - 80.9)	60.4 ± 9.5 58.7 (50.9 - 74.5)	57.2 ± 12.4 56.5 (37.3 - 70.7)
BIC (%)	**Groove**	**1 month**	4.1 ± 4.2 3.7 (0.0 - 8.9)	32.6 ± 5.0 34.3 (26.9 - 38.5)	24.3 ± 22.0 13.0 (4.9 - 48.4)	30.5 ± 20.2 26.8 (8.4 - 57.8)	18.0 ± 14.7 22.0 (0.0 - 36.7)
		3 months	17.4 ± 17.0 17.4 (0.0 - 36.5)	32.7 ± 23.2 34.5 (3.9 - 59.5)	17.4 ± 15.5 10.3 (7.2 - 47.6)	28.2 ± 14.2 20.8 (16.7 - 50.7)	15.1 ± 6.6 12.6 (8.9 - 25.4)
	Thread	**1 month**	28.8 ± 13.6 26.0 (12.2 - 49.4)	48.9 ± 17.9 42.7 (26.8 - 70.2)	46.5 ± 16.2 51.0 (19.3 - 60.6)	47.3 ± 13.0 53.7 (27.9 - 59.3)	32.7 ± 10.3 39.3 (16.8 - 39.8)
		3 months	42.8 ± 13.3 41.8 (23.5 - 64.4)	47.9 ± 14.2 42.2 (35.3 - 71.1)	44.5 ± 14.8 37.4 (32.1 - 65.4)	39.4 ± 8.7 36.9 (28.8 - 52.6)	36.7 ± 10.4 41.6 (20.9 - 47.0)

H2O: distilled water (control); PPA10: polyphosphoric acid 10 wt%; PPL1: phosphorylated pullulan 1wt%; PPL10: phosphorylated pullulan 10 wt%; PPL10 BMP: phosphorylated pullulan 10 wt% + 1 μg BMP-2.

FIGURE LEGENDS

Fig. 1. Surgical protocol and implants. A) Histological section showing the implant design and its osseointegration. Scale bar = 1 mm. B) Details of implant design and its dimensions. The grooved [G] and the threaded [T] parts indicate the regions where histomorphometrical analyses were performed. C) Anatomy of the pig skull: parietal bones (P); frontal bones (F); nasal bones (N). Gray circles show the sites of implantation in the parietal bone. D) Surgical site with the implants inserted in the parietal bone. A second implant per group was inserted in the frontal bone but these were not used due to the extensive pneumatization of the frontal bone at the time of sacrifice.

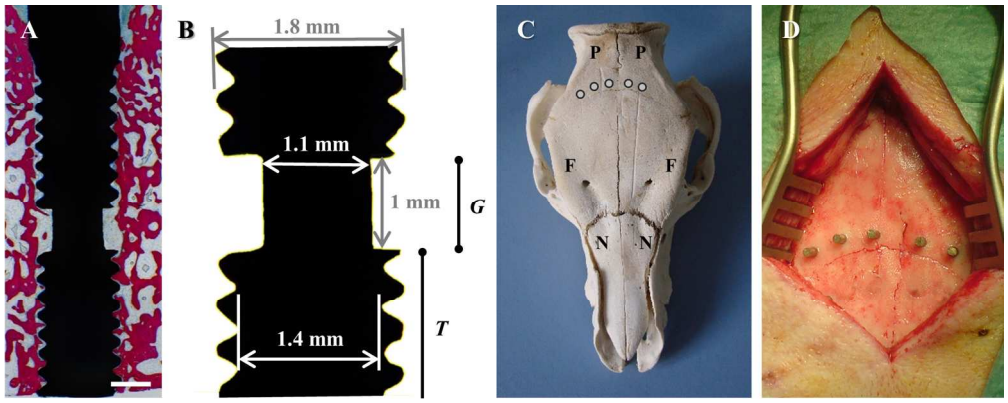
Fig. 2. Representative histological section of the bone-implant interface. A- Peri-implant bone relative to the tissue (B/T): the amount of bone in the peri-implant region up to 100 µm away from the implant surface. B - Same image after being digitally processed for histomorphometrical analysis. C - Bone-to-implant-contact (BIC): summation of the lengths of contact between bone and implant, divided by the implant length under consideration. Scale bar: 250 µm.

Fig. 3. Histological analysis of tissue reaction around the surface of an implant treated with phosphorylated pullulan at a concentration of 10% wt and incorporated with 1 µg of BMP-2 per implant (group PPL10 BMP). Sections were stained with a combination of Stevenel's blue and Von Gieson's picrofuchsin. Note the osteoclast activity around the implant surface (arrows).

Fig. 4. Representative histotlogical sections per experimental group at each healing time (1 month at upper row and 3 months at lower row) showing an overview of the areas of interest (groove and thread): [a and f] water (H2O, control group); [b and g] 10% polyphosphoric acid (PPA10); [c and h] 1% phosphorylated pullulan (PPL1); [d and i] 10% phosphorylated pullulan (PPL10); [e and j] 10% phosphorylated pullulan + 1 µg BMP-2 (PPL10 BMP). Scale bar: 1 mm.

Fig. 5. Results for the peri-implant bone relative to the tissue (B/T, %), quantified at the grooved [G] and the threaded lower [T] part up to 100 µm away from the implant surface. Connectives bars indicates means with significant statistical differences (Mann Whitney U-test with a significance level of 5%).

Fig. 6. Results for bone-to-implant-contact (BIC, %): summation of the lengths of contact between bone and implant/implant length under consideration measured at the grooved [G] and the threaded lower [T] part. Connectives bars indicate means with significant statistical differences (Mann Whitney U-test with a significance level of 5%).



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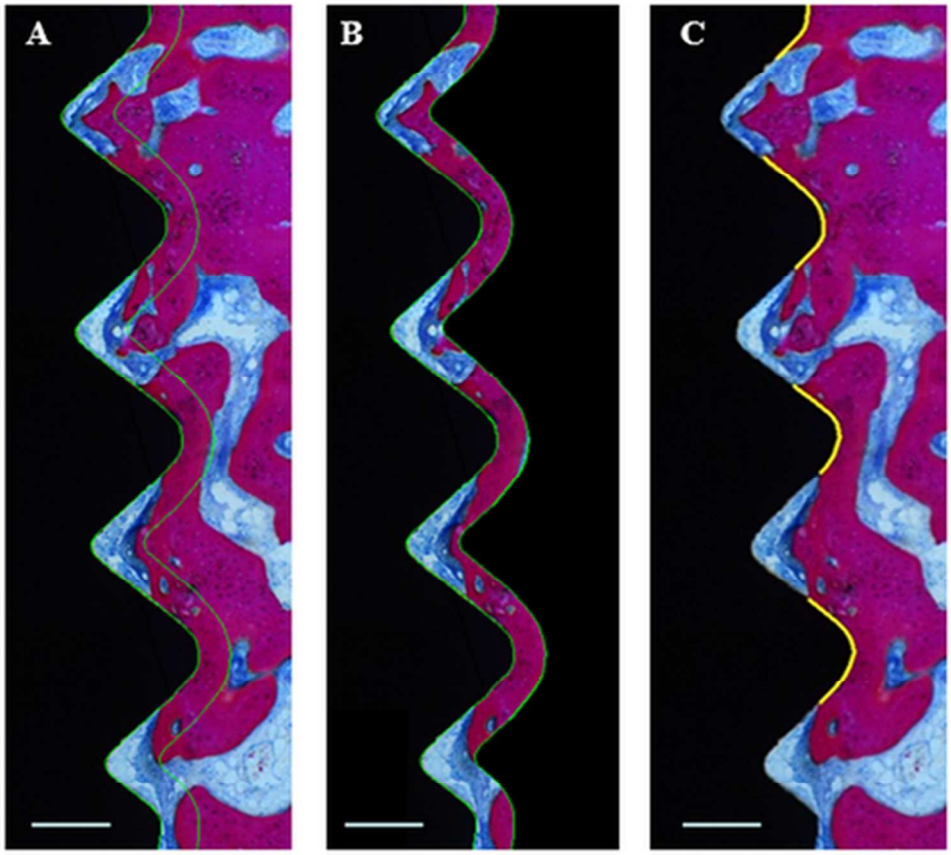

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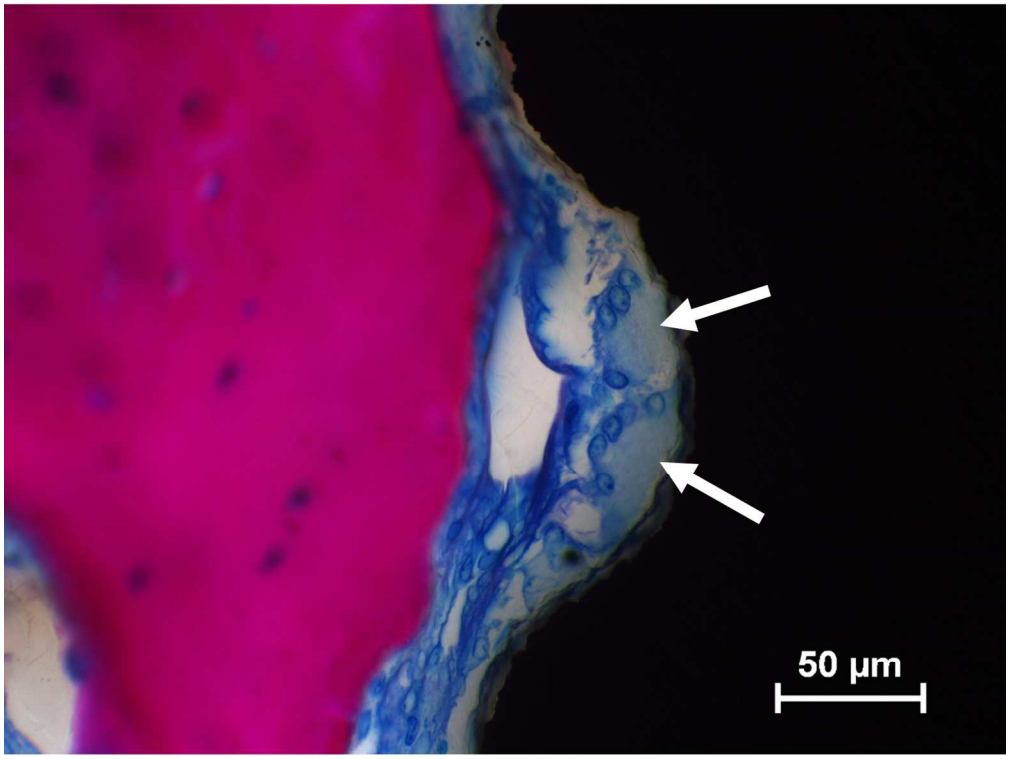

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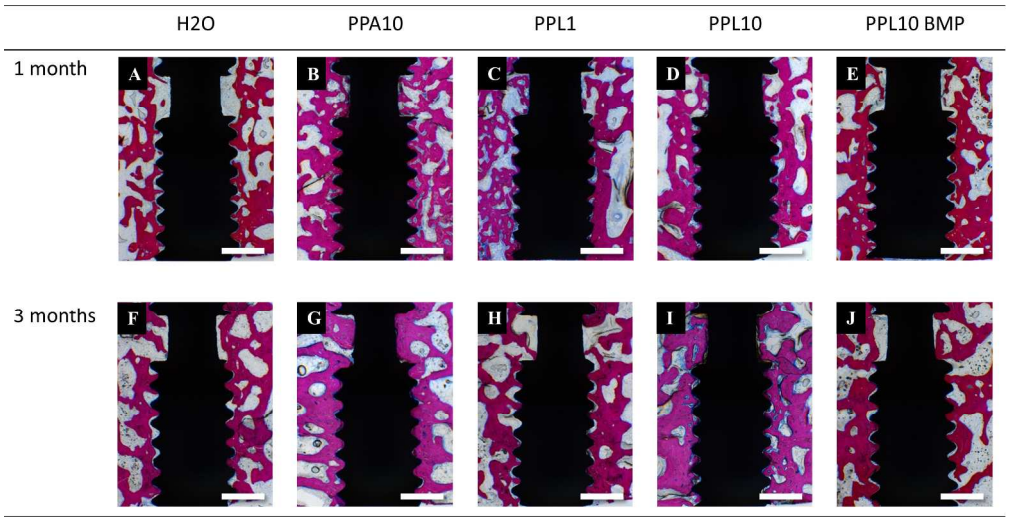

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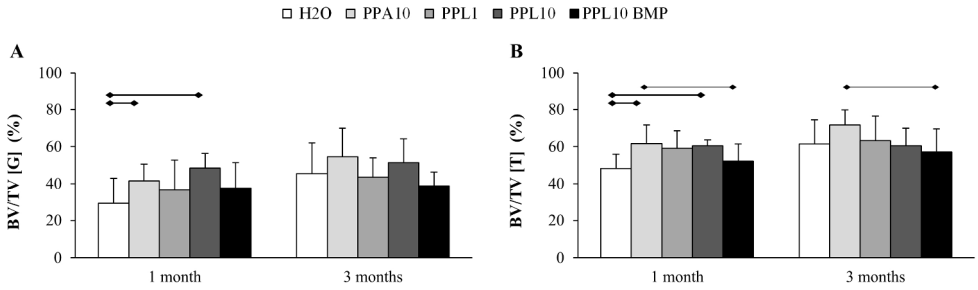

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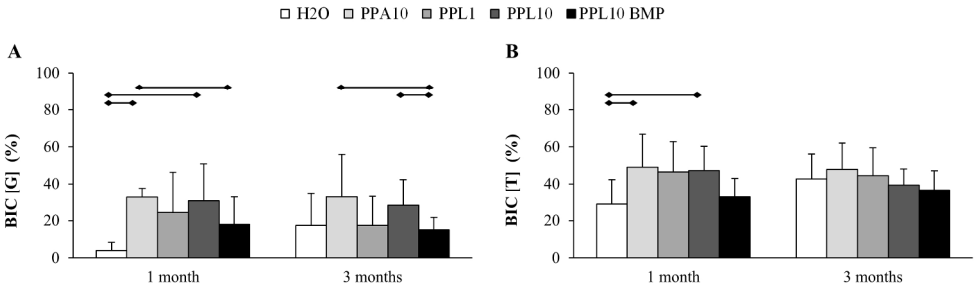

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		3 months	17.4 ± 17.0 17.4 (0.0 - 36.5)	32.7 ± 23.2 34.5 (3.9 - 59.5)	17.4 ± 15.5 10.3 (7.2 - 47.6)	28.2 ± 14.2 20.8 (16.7 - 50.7)	15.1 ± 6.6 12.6 (8.9 - 25.4)
	Thread	**1 month**	28.8 ± 13.6 26.0 (12.2 - 49.4)	48.9 ± 17.9 42.7 (26.8 - 70.2)	46.5 ± 16.2 51.0 (19.3 - 60.6)	47.3 ± 13.0 53.7 (27.9 - 59.3)	32.7 ± 10.3 39.3 (16.8 - 39.8)
		3 months	42.8 ± 13.3 41.8 (23.5 - 64.4)	47.9 ± 14.2 42.2 (35.3 - 71.1)	44.5 ± 14.8 37.4 (32.1 - 65.4)	39.4 ± 8.7 36.9 (28.8 - 52.6)	36.7 ± 10.4 41.6 (20.9 - 47.0)

H2O: distilled water (control); PPA10: polyphosphoric acid 10 wt%; PPL1: phosphorylated pullulan 1wt%; PPL10: phosphorylated pullulan 10 wt%; PPL10 BMP: phosphorylated pullulan 10 wt% + 1 µg BMP-2.

SUPPLEMENTARY MATERIAL

Supporting information for review and online publication only.

Table 1. Results of the Wilcoxon signed-rank test for Bone Fraction (B/T) in the groove area (G) at 1-month healing time (p values).

	H2O	PPA10	PPL1	PPL10	PPL10 BMP
H2O		0.043	0.686	0.043	0.225
PPA10	0.043		0.686	0.080	0.500
PPL1	0.686	0.686		0.225	1.000
PPL10	0.043	0.080	0.225		0.225
PPL10 BMP	0.225	0.500	1.000	0.225	

H2O: distilled water (control); PPA10: polyphosphoric acid 10 wt%; PPL1: phosphorylated pullulan 1wt%; PPL10: phosphorylated pullulan 10 wt%; PPL10 BMP: phosphorylated pullulan 10 wt% + 1 µg BMP-2. Statistically significant differences are highlighted in red when p<0.05.

Table 2. Results of the Wilcoxon signed-rank test for Bone Fraction (B/T) in the groove area (G) at 3-month healing time (p values).

	H2O	PPA10	PPL1	PPL10	PPL10 BMP
H2O		0.345	0.753	0.600	0.463
PPA10	0.345		0.249	0.917	0.116
PPL1	0.753	0.249		0.345	0.345
PPL10	0.600	0.917	0.345		0.173
PPL10 BMP	0.463	0.116	0.345	0.173	

H2O: distilled water (control); PPA10: polyphosphoric acid 10 wt%; PPL1: phosphorylated pullulan 1wt%; PPL10: phosphorylated pullulan 10 wt%; PPL10 BMP: phosphorylated pullulan 10 wt% + 1 µg BMP-2. Statistically significant differences are highlighted in red when p<0.05.

Table 3. Results of the Wilcoxon signed-rank test for Bone Fraction (B/T) in the thread area (T) at 1-month healing time (p values).

	H2O	PPA10	PPL1	PPL10	PPL10 BMP
H2O		0.043	0.138	0.043	0.345
PPA10	0.043		0.686	0.893	0.043
PPL1	0.138	0.686		0.893	0.345
PPL10	0.043	0.893	0.893		0.138
PPL10 BMP	0.345	0.043	0.345	0.138	

H2O: distilled water (control); PPA10: polyphosphoric acid 10 wt%; PPL1: phosphorylated pullulan 1wt%; PPL10: phosphorylated pullulan 10 wt%; PPL10 BMP: phosphorylated pullulan 10 wt% + 1 µg BMP-2. Statistically significant differences are highlighted in red when p<0.05.

Table 4. Results of the Wilcoxon signed-rank test for Bone Fraction (B/T) in the thread area (T) at 3-month healing time (p values).

	H2O	PPA10	PPL1	PPL10	PPL10 BMP
H2O		0.116	0.753	0.753	0.600
PPA10	0.116		0.345	0.116	0.028
PPL1	0.753	0.345		0.753	0.753
PPL10	0.753	0.116	0.753		0.600
PPL10 BMP	0.600	0.028	0.753	0.600	

H2O: distilled water (control); PPA10: polyphosphoric acid 10 wt%; PPL1: phosphorylated pullulan 1wt%; PPL10: phosphorylated pullulan 10 wt%; PPL10 BMP: phosphorylated pullulan 10 wt% + 1 µg BMP-2. Statistically significant differences are highlighted in red when p<0.05.

Table 5. Results of the Wilcoxon signed-rank test for Bone-to-implant Contact (BIC) in the groove area (G) at 1-month healing time (p values).

	H2O	PPA10	PPL1	PPL10	PPL10 BMP
H2O		0.043	0.225	0.043	0.144
PPA10	0.043		0.225	0.686	0.043
PPL1	0.225	0.225		0.500	0.686
PPL10	0.043	0.686	0.500		0.500
PPL10 BMP	0.144	0.043	0.686	0.500	

H2O: distilled water (control); PPA10: polyphosphoric acid 10 wt%; PPL1: phosphorylated pullulan 1wt%; PPL10: phosphorylated pullulan 10 wt%; PPL10 BMP: phosphorylated pullulan 10 wt% + 1 µg BMP-2. Statistically significant differences are highlighted in red when p<0.05.

Table 6. Results of the Wilcoxon signed-rank test for Bone-to-implant Contact (BIC) in the groove area (G) at 3-month healing time (p values).

	H2O	PPA10	PPL1	PPL10	PPL10 BMP
H2O		0.345	0.917	0.345	0.600
PPA10	0.345		0.075	0.753	0.116
PPL1	0.917	0.075		0.173	0.600
PPL10	0.345	0.753	0.173		0.028
PPL10 BMP	0.600	0.116	0.600	0.028	

H2O: distilled water (control); PPA10: polyphosphoric acid 10 wt%; PPL1: phosphorylated pullulan 1wt%; PPL10: phosphorylated pullulan 10 wt%; PPL10 BMP: phosphorylated pullulan 10 wt% + 1 µg BMP-2. Statistically significant differences are highlighted in red when p<0.05.

Table 7. Results of the Wilcoxon signed-rank test for Bone-to-implant Contact (BIC) in the thread area (T) at 1-month healing time (p values).

	H2O	PPA10	PPL1	PPL10	PPL10 BMP
H2O		0.043	0.080	0.043	0.686
PPA10	0.043		0.686	0.500	0.225
PPL1	0.080	0.686		0.893	0.225
PPL10	0.043	0.500	0.893		0.138
PPL10 BMP	0.686	0.225	0.225	0.138	

H2O: distilled water (control); PPA10: polyphosphoric acid 10 wt%; PPL1: phosphorylated pullulan 1wt%; PPL10: phosphorylated pullulan 10 wt%; PPL10 BMP: phosphorylated pullulan 10 wt% + 1 µg BMP-2. Statistically significant differences are highlighted in red when p<0.05.

Table 8. Results of the Wilcoxon signed-rank test for Bone-to-implant Contact (BIC) in the thread area (T) at 3-month healing time (p values).

	H2O	PPA10	PPL1	PPL10	PPL10 BMP
H2O		0.917	0.917	0.600	0.249
PPA10	0.917		0.600	0.249	0.075
PPL1	0.917	0.600		0.917	0.345
PPL10	0.600	0.249	0.917		0.600
PPL10 BMP	0.249	0.075	0.345	0.600	

H2O: distilled water (control); PPA10: polyphosphoric acid 10 wt%; PPL1: phosphorylated pullulan 1wt%; PPL10: phosphorylated pullulan 10 wt%; PPL10 BMP: phosphorylated pullulan 10 wt% + 1 µg BMP-2. Statistically significant differences are highlighted in red when p<0.05.

Table 9. Results of the Mann Whitney U-test comparing results of 1-month vs 3-month healing times (p values) for each parameter (B/T and BIC) and each area of interest (G and T).

	B/T [G]	B/T [T]	BIC [G]	BIC [T]
H2O (1 month vs 3 months)	0.171	0.083	0.400	0.235
PPA10 (1 month vs 3 months)	0.121	0.121	1.000	1.000
PPL1 (1 month vs 3 months)	0.411	0.927	0.784	0.927
PPL10 (1 month vs 3 months)	0.648	0.784	1.000	0.235
PPL10 BMP (1 month vs 3 months)	0.523	0.648	0.927	0.235

H2O: distilled water (control); PPA10: polyphosphoric acid 10 wt%; PPL1: phosphorylated pullulan 1wt%; PPL10: phosphorylated pullulan 10 wt%; PPL10 BMP: phosphorylated pullulan 10 wt% + 1 µg BMP-2; B/T: bone fraction; BIC: bone-to-implant contact; G: groove; T: thread. Statistically significant differences are highlighted in red when $p<0.05$.

RESPONSE TO REVIEWERS MANUSCRIPT ID CPE-09-16-6565 December 28th 2016

Titanium implant functionalization with phosphorylated pullulan at specific concentration favors in vivo osseointegration

First of all we would like to thank the editors for providing us the opportunity to submit a revised version of our manuscript. We would also like to thank the reviewers for their remarks and suggestions. Please find below a point by point answer (in italic) to the comments of the reviewers. The changes in the revised manuscript have been highlighted in red.

Associate Editor

Comment: Abstract - please clearly indicate the primary outcome parameter as well as the Unit of analysis
Answer: *The primary outcome and unit of analysis are now clearly indicated: "As primary outcome, implant osseointegration was evaluated by quantitative histology, namely peri-implant bone formation (B/T in %) and bone-to-implant contact (BIC in %) for each healing period".*

Comment: Clinical Relevance -All sentence in this section appear to be incomplete and require a proper revision.
Answer: *Sentences were rewritten as such:*
"Scientific rationale for the study: To investigate the influence of different implant surface treatments on the efficiency of bone regeneration in a simulated clinical situation.
Principal findings: Both PPA10 and PPL10 seem to induce faster peri-implant osseointegration and bone regeneration. Use of PPL as a carrier for BMP-2 was not efficient on stimulating peri implant bone formation.
Clinical implications: PPA10 and PPL10 may promote more favorable conditions for early implant loading, particularly in unfavorable clinical situations."

Comment: Introduction - please further elucidate the experimental approach and rationale, as well as its relevance to human biology.
Answer: *These aspects have been further elucidated, starting from the relevance to human biology and then the related rational for the experimental approach. In the section "Introduction", we expose the clinical problem, and subsequently present a possible solution, the state of the art in this specific field and how the remaining specific doubts could be addressed, finally describing the experimental approach and the purpose of the study.*

Comment: Materials and Methods:

- "Animal model, implant and surgical protocol" need to be presented in separate sections
- please refer to the ARRIVE guidelines when reporting on experimental studies performed in animals
- please indicate when the study - and in particular the animal experiment - has been carried out
- information on housing and husbandry is missing
- conditions and welfare-related assessments and interventions are missing

Answer: *All items requested are now described in a separate sections named "Animal model", "Implants", and "Surgical Protocol" section.*
We have referred to the ARRIVE guidelines in the sections "Animal model" and "Surgical Protocol". All items have been reported accordingly, including timeline of the study and animal experiment, housing and husbandry information, conditions and welfare-related assessment and interventions.

Comment: Materials and Methods: Please explain how the number of animals was arrived at
Answer: *According to Pearce et al (2007), international standards stablish that at least 2 animals should be used for each treatment at each healing time when implantation of biomaterials in bone of pigs is considered. Previous research using*

the same methodology and implantation site (Lutz et al, 2008) showed that the use of 4 animals were sufficient to assure statistical significance. To compensate possible drop-outs, while minimizing the number of animals, the sample size per group was estimated to be n = 6, i.e. 12 animals in total. This number has been confirmed to be sufficient in some of our previous research in which the same methodology was employed (Cardoso et al, 2016). This is now explained in the last paragraph of the "Animal model" section.

Comment: Materials and Methods: Give full details of how sites were allocated to experimental groups, including randomisation or matching if done.
Answer: *Sites of implantation were randomly allocated to experimental groups using a computer-generated list (www.randomization.com) with a 1:1 allocation using random block size of 5. This is now mentioned in the section "Surgical protocol".*

Comment: Materials and Methods: Primary and secondary outcomes must be clearly defined.
Answer: *Primary and secondary outcomes are now clearly defined in the last paragraph of the "Specimen preparation and analysis" and the "Statistical analyses" sections of Materials and Methods, as mentioned below:*
"The primary outcome was defined as the efficiency of different titanium surface treatments (H2O, PPA10, PPL1, PPL10, PPL10 BMP) on bone regeneration at 2 specific healing times, namely 1 and 4 months. The primary outcome variable was bone density (B/T in %) and bone-to-implant contact (BIC in %) within the two regions of interest, i.e groove (G) and thread (T). The secondary outcome was to define the efficiency of these surface treatments on bone regeneration over time (1-month vs 3-month healing times)."
"The Wilcoxon signed-rank test was performed at a significance level of 5% … for each of the selected parameters (B/T and BIC; primary outcome)".
"The results obtained for the 1 month and the 3 months groups were also compared (secondary outcome) using … the Mann Whitney U-test".

Comment: Materials and Methods: calibration/ blinding of the examiner/s is missing
Answer: *All surgical procedures were performed by the same calibrated operator. This is now mentioned in the section "Surgical protocol". All histological and histomorphometrical analyses were performed by the same blinded and calibrated examiner. This is now mentioned in the section "Specimen preparation and analysis".*

Comment: Materials and Methods: Histological analysis - how many samples were stained and evaluated per experimental site?
Answer: *One single sample, the most central one, was stained and evaluated per experimental site. This is mentioned in the section "Specimen preparation and analysis".*

Comment: Materials and Methods: Statistics: how did the authors account for multiple-testing?
Answer: *Multiple testing was used to increase the sensitivity of the statistical analysis as the global number of comparisons is too large. In other words, we opted for an exploratory analysis so that statistically significant differences would become more evident.*
On the other hand, the specific non-parametric tests employed (Wilcoxon signed-rank test and Mann Whitney U-test) comply with 2 different criteria of dependency between data sets.
When comparing the performance of different surface treatments at the same healing time (1 or 3 months), the statistical analysis should take into account the dependency between data sets as implants from the 5 different groups were installed in the same animal (experimental unit). In this case, the Wilcoxon signed-rank test was used.
On the other side, when comparing the performance of the different surface treatments over time (1 vs 3-month healing), independency between data sets should be considered as implants were installed in differences animals. In this case, Mann Whitney U-test was used.
This reasoning is now mentioned in the section "Statistical analysis".

We also noticed that the results of the Mann Whitney U-test were not described in the Results. This is now mentioned accordingly in the last paragraph of the chapter "Results".

Comment: Discussion: Statistics: please discuss why the implants have not been placed in the mandible - what is the clinical relevance of data collected in the parietal bone?

Answer: *As clarified in the 2nd paragraph of the Discussion, "domestic pigs show considerable bone similarities to human beings, including important aspects such as osseous macrostructure, microstructure, composition and bone remodeling process and rate, therefore representing a suitable option for studies involving implants (Pearce et al. 2007). The calvaria was chosen due to its structural similarity to the maxillary region while allowing for a more controllable and reproducible surgical procedure when compared to protocols involving intraoral surgery (Lutz et al. 2008)".*

By opting for the parietal bone, we avoided external factors that could interfere with the osseointegration and overshadow the effect of the proposed treatments, such as difficulties during the surgical procedure, contamination, movement of remaining teeth and damages in the surgical area due to masticatory function.

Comment: Figures: lower magnification views should also be included for all groups.

Answer: *Figure 4 was included with lower magnification views of representative images of the areas of interest (groove and thread), being one per experimental group at each healing time. This figure has been mentioned in the text in the section Results/Histological findings.*

Editor in Chief Comments.

Comment: A critical question needs to be addressed: lots of studies are attempting to modify titanium in order to improve its biological performance. Many of these studies are published in more specialized Journals. Why should JCP publish this specific report? Does it have a significantly different potential for clinical application? How does this relate to many other approaches being investigated?

Answer: *Our decision to submit this manuscript to JCP was indeed based on the scope of this periodic. The first section of the Author's guidelines mentions that "JCP publishes original contributions on high scientific merit in the fields of periodontology and implant dentistry". Our research explores important aspects in both fields. Specifically about of the scope of the Journal, it is clear that it "encompasses a lot of specific fields, among then, tissue osseointegration of dental implants, bone healing and regeneration, the clinical aspects of tooth replacement with dental implants and finally, advances in implant techniques and procedures", which closely reflects the scope of our research.*

Regarding to the potential for clinical application, our experiment evaluates different implant surface treatments with direct potential for clinical application. This stage of maturity in our research line is a result of many successful previous exploratory studies involving:

- *surface characterization of titanium treated with different concentrations of organic and inorganic phosphate-containing polymers;*
- *in vitro mesenchymal cell behavior on titanium treated with these polymers;*
- *and in vivo proof of concept involving small animal models in less clinically significant set-ups.*

The aim of the current study is to contribute with a clinically relevant information and provide different options to improve the speed and the quality of the osseointegration.

Comment: are there potential conflict of interest that need to be disclosed?

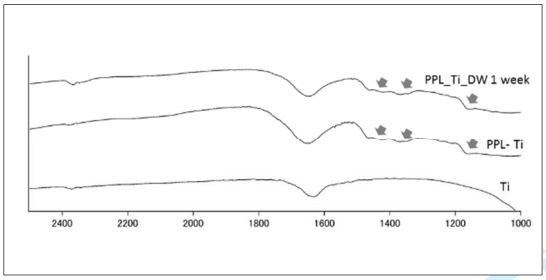
Answer: *As stated in the Wiley's Conflict of Interest Form, there is no conflict of interest to be disclosed.*

Referee 1 Comments.

Comment: The implant has 2 different diameters, thus the smaller one may prevent risk for the coat to be detached at least in that area during installation but do the authors have any information on the stability of the coat? Durability?

Answer: *Thanks to its unique linkage pattern, pullulan offers interesting physical properties, including adhesive ability and the capacity to form thin and transparent biodegradable films (Cheng, Demirci & Catchmark, 2011). On the other hand, pullulan films are highly soluble (Tong, Xiao & Lim, 2008), leading to a fast release of phosphorylated polymers into the regeneration area.*

To evaluate if any phosphorylated pullulan remains on the titanium surface after the solubility of the coating, we used a Shimadzu IRAffinity-1 FTIR Spectrophotometer (Shimadzu, Japan). As shown in the Figure below, the FTIR spectra of PPL-treated titanium revealed three absorption bands around 1000-1250 cm−1, which were not present in the FTIR spectra of pure titanium. These three absorption bands are attributed to PPL. These peaks remained present even after active washing and storage in water for 1 week. These results confirm the presence of a stable layer of PPL on the treated Ti surfaces.



This chemical characterization was used in another study which, in the meantime, has been accepted for publication (Cardoso et al., 2017). Therefore, this can now be used as a reference in the present study, as included in the 3rd paragraph of the section Discussion. It remains, however, unknown if the positive effect of PPL is due to the phosphorylated polysaccharides which are released in the regeneration area, or due to the molecules that remain on the titanium surface.

Finally, implants treated with PPL10 showed higher values of bone formation (B/T) and implant osseointegration (BIC) when compared to the non-treated implants (H2O) after 1 month irrespective of the site to be considered (thread or groove). This shows the positive effect of PPL irrespective of the mechanical challenge during the insertion of the implant in the bone. This is now also discussed in the 7th paragraph of the Discussion chapter.

Comment: Did the coat influence the surface topography on a micro or nano level?

Answer: *The topography of the implants is indeed an important aspect to be taken into account. According to the 4th paragraph of the section Discussion, "A hierarchical surface topography including both micron-scale and submicron-scale structures seems to be able to promote an enhanced osteogenic effect (Zinger et al. 2005). In our study, a machined implant surface devoid of complex topographic structures has been employed to avoid that such physical aspects would overshadow the effect of the chemical surface modifications, which represent the main object of this study. As previously presented, no topographical modification can be expected after treatment of titanium surfaces with PPA and PPL in either micro or nanometrical level (Cardoso et al. 2014), suggesting that all samples employed displayed the same morphology and roughness features irrespective of the treatment to which they were submitted".*

Small modifications in the text have been done to emphasize that PPL and PPA are not able to modify the surface topography in either micro or nanometrical level.

Comment: The title do not correspond very well with the achieved results, more correct would be "…may favor early bone formation…"

Answer: *The title has been adapted according to the reviewer's suggestion.*

Referee 2 Comments.

Comment: Both polyphosphoric acid at 10% and phosphorylated pullulan at 10% achieved better results in comparison to the control with water. Why is in the entire manuscript only the effect of phosphorylated pullulan discussed? Why it is only concluded that phosphorylated pullulan rendered a positive effect on the osseointegration? Based on the results it should be concluded that both polyphosphoric acid at 10% and phosphorylated pullulan at 10% stimulate the osseointegration.

Answer: *We have emphasized the role of PPL on osseointegration as the role of PPA has already been reasonably explored in some of our previous studies (Maekawa et al., 2007, 2008 and 2009). Differently form PPA, PPL presents specific characteristics which allows it to be used as a carrier for drugs or growth factors, as explored in the present research.*

Anyway, the function of PPA on osseointegration is presented in the 2nd and 3rd paragraphs of the section "Introduction", and in many passages throughout the discussion.

The conclusion sections have been rephrased as indicated by the reviewer, including PPA 10 wt%:

Conclusion at the Abstract: "Functionalizing titanium implants with inorganic or organic phosphate-containing polymers at 10 wt% concentration may stimulate peri-implant bone formation and implant osseointegration at early healing times".

Conclusion in the main manuscript: "Phosphate-containing polymers, whether in their inorganic or organic form, may induce extended peri-implant bone formation and osseointegration in early phases of the healing process in a dose-dependent manner. Both PPA 10 wt% and PPL 10 wt% promoted higher values of B/T and BIC at 1-month healing time. After 3 months of healing, however, PPA and PPL seem not to play a role on histomorphometrical outcomes".

Comment: The differences between these two substances (polyphosphoric acid and phosphorylated pullulan) need further explanation.

Answer: *As mentioned in the manuscript, PPA is an inorganic polymer while PPL is an organic polymer. Differently from PPA, which is artificially developed, pullulan is all natural while modified to include phosphorus into the polymer chain. However, in the context of the present study, the main difference between these two substances is that, as a polysaccharide, PPL can form a film on the treated surface which can be further investigated for the immobilization of growth factors or gene therapy. Actually, this is also the reason why this manuscript focus more on the effects of PPL than PPA. The potential effects of phosphate groups on bone regeneration are well stablished. The purpose of our research line is to advance one step further and investigate the use of PPL as a carrier. We started showing the potential of phosphate groups on cell attachment, proliferation and differentiation (Maekawa et al, 2007 and 2008), and went further with a "proof of concept" study, showing that phosphate based polymers (inorganic and organic) are both able to stimulate bone regeneration in an animal model (Cardoso et al., 2014). At the moment, our work investigates the use of PPL in clinically relevant situations to then further develop on its potential use as a biological carrier for growth factor or drug delivery, as investigated in other areas of medicine (Morimoto et al. 2005, Kato et al. 2007, Prajapati et al. 2013). The present study already initiates this next phase of our research line by investigating the possible use PPL as a carrier for BMP-2.*

All these aspects are already present in the Introduction section. We have made some small modifications in the text to emphasize the difference between PPA and PPL (4th paragraph of the Introduction).

Comment: As far as the interpretation is concerned, it is questionable whether the film-building pullulan had any effect. Indeed, polyphosphoric acid alone rendered the highest mean values. This fact should be discussed.

Answer: *From the statistical point of view, no significant difference was observed between polyphosphoric acid and phosphorylated pullulan in terms of bone regeneration and osseointegration, irrespective of concentration, area of interest or healing time. Therefore, we completely agree with the reviewer: the positive effect of PPL10 cannot be ascribed to its film-building characteristic. We have now made it clear in the 7th paragraph of the Discussion section. Once both PPA and PPL present similar positive effect on bone regeneration, the film-building feature of PLL remains a potential advantage over PPA as it may work as a potential carrier for drug delivery or gene therapy. We already started exploring this aspect in the present study by combining PPL with BMP-2. Further studies are necessary in this sense, as exposed in the last sentences of the section Conclusion.*

Comment: 2. P-values should be reported in the Results section. It is recommended to include the tables containing the results of the descriptive analysis (means, medians, SD, ranges) and the exact P-values.
Answer: *P values are now reported in the Results section ($p>0.05$ or $p<0.05$). Table 1 was included and contains the results of the descriptive analysis (means, SD, medians and ranges). Because we opted for multiple testing in the statistical analyses to compensate the large number of comparisons, it becomes difficult to present all exact P-values in the manuscript itself. Therefore, to fulfill the request of the reviewer, we prepared 9 Tables will all p values as Supplementary Material, "Supporting information for review and online publication only", as suggested in the Guidance for Authors of Journal of Clinical Periodontology.*

Comment: 3. For better understanding, it is recommended to use entire sentences in the chapter Clinical relevance.
Answer: *The chapter "Clinical Relevance" has been rewritten according to the suggestion of the reviewer, as presented below:*
"Scientific rationale for the study: To investigate the influence of different implant surface treatments on the efficiency of bone regeneration in a simulated clinical situation.
Principal findings: Both PPA10 and PPL10 seem to induce faster peri-implant osseointegration and bone regeneration. Use of PPL as a carrier for BMP-2 was not efficient on stimulating peri implant bone formation.
Clinical implications: PPA10 and PPL10 may promote more favorable conditions for early implant loading, particularly in unfavorable clinical situations."

Comment: 4. An image of the experimental region would be valuable, since the applied model with implant placement in porcine skull is not widely used.
Answer: *We included two images in Fig 1 (C and D). Fig 1C shows the anatomy of a pig skull and Fig 1D shows the surgical site with the implants inserted in the parietal bone. Text and Figure legends were adapted accordingly.*

Comment: 5. The number of implants, their bilateral distribution and the distance between the implants need to be clearly described.
Answer: *This information is now described in the "Surgical protocol" section: "Five implantation sites were drilled in a transversal sequence in the parietal bones 0.6 mm far from the coronal suture (Fig. 1C and 1D). Two of these sites were drilled on the right parietal bone while the other three on the left parietal bone, always keeping a distance of about 12 mm between adjacent drilling sites". The allocation of the experimental groups per site is now also described in the same section: "Sites of implantation were randomly allocated to experimental groups using a computer-generated list (www.randomization.com) with a 1:1 allocation and random block size of 5".*

Comment: 6. Conclusions: "Phosphate-containing polymers, whether in their inorganic or organic form...". Can a polymer be inorganic? "...when clinically relevant aspects are taken into account". This part of the sentence is not clear.
Answer: *Indeed, a polymer can be both organic and inorganic. There are many polymers which do not have Carbon in their backbone. Their backbones are made of atoms such as silica, boron, sulphur, etc. Similarly, phosphorus atoms can also form the backbone of a polymer, as in case of polyphosphoric acid (PPA). When two orthophosphoric acid molecules are condensed into one molecule, pyrophosphoric acid ($H_4P_2O_7$) is obtained. Further orthophosphoric acid molecules can be condensed into this molecules one by one to form polyphosphoric acid. Polyphosphoric acid molecules can have dozens of such phosphoric units bonded in a row.*

Throughout the text, more specifically in the Introduction and Discussion sections, we mention that one of the purposes of this study is to evaluate the effect of the studied treatments in a "more clinically relevant situation" as these treatments have already proven to be efficient in some of our previous studies involving cell-culture (Maekawa et al, 2007 and 2008) and animals in a "proof of concept" methodology (Maekawa et al., 2009, Cardoso et al., 2014). In the present study, the clinical relevance lays on the fact that the implants are actively screwed into the bone and that the used animal model closely simulates the bone metabolism of human beings (Pearce et al., 2007).
To avoid misinterpretations, this sentence was deleted from the Conclusion section.

Comment: 7. „Additional studies should define the ideal concentration of BPM-2 to be incorporated in phosphorylated pullulan solutions aiming at enhanced bone healing. Alternatively, further modifications in the physical properties of PPL are necessary before it can be promoted as a carrier for BMP-2 in bone regeneration applications. It is recommended to move these sentences to the Discussion section and to limit the Conclusions to the outcomes of the study.
Answer: *As suggested by the reviewer, these sentences were deleted from the conclusions and are part of the "Discussion" chapter (last sentences of the last paragraph).*
To avoid misunderstanding, we have clarified in the Conclusion chapter that "The association of PPL10 and BMP-2 was not successful on promoting improved bone formation and implant osseointegration as far as the concentrations and conditions defined in this study are taken into account."

Comment: 8. Some images are too small and cannot be assessed
Answer: *We have reassessed the images so that they are presented in sufficient size/resolution.*

Referee 3 Comments.

Comment: The input of animals and effort was immense; the outcome not that spectacular. Especially after 3 month no beneficial effect is seen by any surface modification.
Answer: *Statistically significant differences were found at 1-month healing time, showing that both PPL10 and PPA10 may play a positive role in improving bone regeneration. The fact that no beneficial effect was found at 3-month healing time does not concern us as the osseointegration process is mostly critical in early healing stages or in cases of local or systemic challenges. To emphasize this aspect, we have included the following text in the 5th paragraph of the Discussion: "This suggests that such treatments will not necessarily improve osseointegration in a long term, but they are able to accelerate bone formation in earlier healing stages. Such faster regeneration is highly desirable as it may shorten unloaded healing periods, besides rendering immediate loading protocols more successful and predictable. It is indeed in the early stages of bone regeneration that bone-implant integration is more susceptible to loading challenges (Gapski et al. 2003). A faster regeneration may also be useful in cases of low bone quality due to compromised systemic conditions or site-related bone defects".*

Comment: Surface treatment should be described in great detail.
Answer: *Surface treatment is now described in full details in the section "Implants" of "Materials and Methods". To comply with a reasonable lengthening of the text, the synthesis of phosphorylated pullulan has not been described in detailed. Alternatively, we opted to refer to another study in which this process is fully described. This can be found in the text as such: "The synthesis of PPL was processed as previously described in detail by Cardoso et al. (2014)".*

Comment: The source and characterization of PPA is missing.

Answer: *This is now mentioned in the section "Implants" of "Materials and Methods": "Treatment of the implants with PPA10 was done by immersion in 10 wt% PPA solution for 24 h at 37°C. This solution was obtained by dissolving 10 g of 100% PPA (Merck Schuchardt, Hohenbrunn, Germany) in 100 ml of distilled water".*

Comment: How much PPA or PPL was bound?

Answer: *The chemical analysis of Ti surfaces treated with different concentrations of PPA has been evaluated and presented in one of our previous publications (Maekawa et al., 2007). By using XPS, we found that the average P/Ti ratio of the treated titanium surfaces increased significantly following a PPA concentration dependency. Significantly higher P/Ti ratios were observed on surfaces treated with polyphosphoric acid at 10 wt% as compared to lower concentrations (0,1 and 1 wt%). In this study, it was observed that PPA10 induced higher mesenchymal cell proliferation than its PPA in lower concentrations.*

The chemical analysis of Ti surfaces treated with PPL10 has been evaluated using a Shimadzu IRAffinity-1 FTIR Spectrophotometer and presented in one of our previous publications (Cardoso et al., 2017, in press). The FTIR spectra of PPL-treated titanium revealed absorption bands attributed to PPL. These peaks remained present even after active washing and storage in water for 1 week. These results confirm the presence of a stable layer of PPL on the treated Ti surfaces. This is now mentioned in the 7th paragraph of the section "Discussion".

Quantification of bounded PPA and PPL was measured and included in the text as described below:

- *Materials and Methods: last paragraph of the "Implants" section;*
- *Results: "Spectrophotometric Measurement" section;*
- *Discussion: 7th paragraph*

"The Spectrophotometric measurements in this study also confirm the stable adsorption of phosphate-containing polymers on titanium in a concentration dependent manner, i.e. higher concentrations of PPL did lead to a higher adsorption on the treated surface".

- *Discussion: 6th paragraph*

"Indeed, our Spectrophotometric measurements showed that treatment with PPA10 leads to a noticeably higher adsorption of phosphate groups on titanium than its lower concentration version (PPA1)."

Comment: Was the bound BMP still bioactive?

Answer: *To assure that BMP-2 was still bioactive, it was reconstituted as per the instructions of the manufacturer. The implants were treated with the reconstituted protein at room temperature in sterile environment and then implanted immediately once dried. Care was taken not to contaminate the protein or to change the environmental temperature to denature the protein. This is now mentioned in the section "Implants" of "Materials and Methods".*

In one of our previous studies in which the same methodology was used (Chaudhari et al., 2013), the BMP-2 bound to titanium surface was kept in cell culture medium. The protein released to the cell culture medium was then measured using ELISA. Binding of these released protein to the ELISA plate shows that the protein was still bioactive.

Comment: Why use pullulan when PPA works also efficiently?

Answer: *As mentioned in the manuscript, PPA is an inorganic polymer while PPL is an organic polymer. Differently from PPA, which is artificially developed, pullulan is all natural while modified to include phosphorus into the polymer chain. However, in the context of the present study, the main difference between these two substances is that, as a polysaccharide, PPL can form a film on the treated surface which can be further investigated for the immobilization of growth factors or gene therapy. Actually, this is also the reason why this manuscript focus more on the effects of PPL*

than PPA. The potential effect of phosphate groups on bone regeneration is well stablished. The purpose of our research line now is to advance in this subject and investigate the use of PPL as a carrier. We started showing the potential of PPA on cell attachment, proliferation and differentiation (Maekawa et al, 2007 and 2008), and went further with a "proof of concept" study, showing that phosphate based polymers (inorganic and organic) are both able to stimulate bone regeneration in an animal model (Cardoso et al., 2014). At the moment, our work investigates the use of PPL in clinically relevant situations to then further develop on its potential use as a biological carrier for growth factor or drug delivery, as investigated in other areas of medicine (Morimoto et al. 2005, Kato et al. 2007, Prajapati et al. 2013). The present study already initiates this next phase of our research line by investigating the possible use PPL as a carrier for BMP-2. All these aspects are already present in the Introduction section. We have made some modifications in the text to explain the difference between PPA and PPL and to emphasize the advantages of PPL (4th paragraph of the Introduction). Once both PPA and PPL present similar positive effect on bone regeneration, the film-building feature of PLL remains a potential advantage over PPA as it may work as a potential carrier for drug delivery or gene therapy. We already started exploring this aspect in the present study by combining PPL with BMP-2. Because the results were not so promising in this sense, further studies are necessary to explore this field of expertise, as exposed in the last sentences of the section Conclusion.

Comment: On PPA work of WEG Müller has to be cited. He has also shown positive effect of PPA on bone regeneration.
Answer: *The work of WEG Muller is indeed outstanding and has been cited accordingly (3rd paragraph of the Discussion chapter):*
"Finally, Muller et al. (2015) has recently shown that polyphosphate stimulates osteoclast-like cells by promoting a significant increase in the levels of intracellular and extracellular ATP, thus functioning as a "metabolic fuel" for hydroxyapatite formation on the plasma membranes of osteoblasts".

Comment: The BIC-figure is too small to be evaluated properly.
Answer: *We have reassessed the images so that they are presented in sufficient size/resolution.*

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